



Europäisches
Patentamt

European
Patent Office
EP 99/5476

Office européen
des brevets

REC'D 07 OCT 1999

WIPO PCT

4

Bescheinigung

Certificate

Attestation

Die angehefteten Unterla-
gen stimmen mit der
ursprünglich eingereichten
Fassung der auf dem näch-
sten Blatt bezeichneten
europäischen Patentanmel-
dung überein.

The attached documents
are exact copies of the
European patent application
described on the following
page, as originally filed.

Les documents fixés à
cette attestation sont
conformes à la version
initialement déposée de
la demande de brevet
européen spécifiée à la
page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

98202594.2

**PRIORITY
DOCUMENT**
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

I.L.C. HATTEN-HECKMAN

DEN HAAG, DEN
THE HAGUE, 29/09/99
LA HAYE, LE

THIS PAGE BLANK (USP)



Europäisches
Patentamt

European
Patent Office

Office européen
des brevets

**Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation**

Anmeldung Nr.:
Application no.:
Demande n°: 98202594.2

Anmeldetag:
Date of filing: 31/07/98
Date de dépôt:

Anmelder:
Applicant(s):
Demandeur(s):
Akzo Nobel N.V.
6824 BM Arnhem
NETHERLANDS

Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:
Attenuated herpesvirus

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat:
State:
Pays:

Tag:
Date:
Date:

Aktenzeichen:
File no.
Numéro de dépôt:

Internationale Patentklassifikation:
International Patent classification:
Classification internationale des brevets:

C12N15/38, C12N7/04, A61K39/245, A61K39/27

Am Anmeldetag benannte Vertragsstaaten:
Contracting states designated at date of filing: AT/BE/CH/CY/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE
Etats contractants désignés lors du dépôt:

Bemerkungen:
Remarks:
Remarques:

THIS PAGE BLANK (USPTO)

THIS PAGE BLANK (USPTO)

31. 07. 1998

ATTENUATED HERPESVIRUS

The present invention relates to a new Equine herpesvirus (EHV) mutant, to its use in vaccines, to DNA sequences and vectors harboring a mutated EHV sequence, to host cells transfected with such vectors and to a
5 method of attenuating pathogenic micro-organisms in general, such as herpesviruses, and EHV in particular.

Equine herpesviruses comprise a group of antigenic distinct biological agents which cause a variety of infections in horses, ranging from subclinical
10 to fatal disease.

The Equine herpesvirus is inter alia one of the most common causes of infectious abortions and may account for 15% of all mare abortions that occur during the last six months of pregnancy. Equine herpesvirus type
15 1 (EHV-1) can cause abortion, perinatal foal mortality, respiratory inflammation and neurologic disease.

Although very similar to EHV-4 (previously classified as EHV-1 subtype 2), EHV-1's main clinical sign is abortion while EHV-4 results in respiratory
20 disease. But both cross over and can be clinically indistinguishable as a disease.

The virus is contracted through inhalation. The respiratory signs can range from severe to inapparent. Abortion occurs primarily in mares over 7 months pregnant, follows infection in 14 to 120 days and can occur
25 suddenly without signs in the mare. The virus infects fetal lung and liver tissue and the mare placental endothelial tissue. Abortion may occur because of direct effects on the fetus or because of placental separation.
30 Near term fetuses may be born alive but die quickly due to lung pathology.

Primary infection of upper respiratory tract of young horses results in a febrile illness which lasts for 8 to 10 days. Immunologically experienced mares may be
35 reinfected via the respiratory tract without disease becoming apparent so that abortion usually occurs without warning. The neurological syndrome is associated with respiratory disease or abortion and can affect animals of

either sex at any age, leading to incoordination, weakness and posterior paralyses.

Other EHV viruses are EHV-2 or Equine Cytomegalovirus, which is a ubiquitous antigenically heterogeneous, usually slowly growing group of viruses, causing no-known disease, and EHV-3, the Equine Coital exanthema virus which is the causative agent of a relatively mild pregenital exanthema of both mare and stallion. EHV-1 subtype 2 is now called EHV-4, and is primarily associated with respiratory disease, although sporadic EHV-4 induced abortions have been reported.

The genomic structure of the EHV is similar to that of other alpha herpesviruses, comprising a double stranded linear molecule consisting of two covalently linked segments (U_L and U_S), wherein the U_S segment is flanked by inverted repeats. Of the EHV EHV-1 is the most extensively studied. Telford, E.A.R. et al., Virology 189, 304-316 (1992) have published the complete DNA sequence of EHV-1. The genome consists of about 150,000 bp and about 76 distinct genes have been recognized up to now.

Equine herpesviruses in general and EHV-1 in particular, are ubiquitous pathogens in horses. EHV-1 can even cause epidemics of abortion, respiratory tract disease and central nervous system disorders. Prevention of infection with the virus is therefore of major economic importance, because EHV can be a severe threat, especially to horses living in close groups, such as studs.

Current vaccines against these viruses comprise chemically inactivated viruses or attenuated live viruses. However, these require multiple administration and have only a limited efficacy.

Inactivated vaccines general induce only a low level of immunity. Attenuated live vaccines are thus preferred because they evoke a more long-lasting immune response and are easier to produce. Attenuation can be obtained by serial passages of virulent strains in tissue

culture of other hosts than the natural one. However, the strains thus obtained are not very well defined and were considered not to be effected. Furthermore, there is always a risk that the viruses revert to virulence
5 resulting in disease in inoculated animals. For this reason genetic attenuation was adopted as a novel approach to obtain safe vaccine strains.

Genetic attenuation consists for example of deletion of one or more non-essential genes. Examples are
10 viruses with deletions in the thymidine kinase gene or the gene encoding glycoprotein gE. Both have been used successfully for genetic attenuation of herpesviruses in general but similar mutations in EHV-1 strongly abolished replication in the host and made the viruses of no longer
15 use for vaccine purposes.

It is therefore a first objective of the present invention to provide new EHV virus strains that do not have the drawbacks mentioned above when used in vaccines.

20 It is a second objective of the invention to provide a method for the more general genetic attenuation of herpesviruses.

The third objective of the invention is to provide for live vaccines that comprise said novel EHV
25 mutant viruses.

The first objective is achieved by an Equine herpesvirus (EHV) mutant, comprising one or more deletions, substitutions or insertions in the promoter region of an essential viral gene.

30 Because attenuation of EHV by mutating non-essential genes was not successful, the skilled person would have considered the mutation of essential genes to be even less promising since one would not have expected the virus to replicate in the host at all. Therefore, the
35 finding of the invention that the promoter region of essential genes can be mutated to reduce the virulence of EHV viruses leading to a successful attenuation is highly surprising.

In a first embodiment of the invention the promoter region is of the immediate early (IE) gene of the EHV virus.

Other essential genes of which the promoters can be used are the genes involved in DNA replication (e.g. gene 57 in EHV-1 encoding the helicase/primase complex), or transactivators of immediate early genes such as gene 12 in EHV-1 or genes encoding essential structural components of the virion such as the glycoproteins gB, gH and gL in EHV-1 or other herpesviruses.

The sequence upstream of the transcription initiation start contains several elements that regulate expression of a gene. All these elements together are defined as the promoter. The core sequence of the promoter also known as TATA-box is located at about 40 base pairs from the transcription start although this structure may vary considerably from case to case. Other more or less conserved elements are located further upstream such as the CCAAT sequence and CG repeated motives. Some elements are specifically found in certain types of promoters such as a TAATGARATTC consensus sequence in immediate early genes of herpesviruses. Also the sequence corresponding to the non-translated region upstream of the ATG start codon can be involved in promoter activity. Many of these elements have not yet been defined nor is fully understood how they interact with the so-called transcription factors as a result of which transcription of the gene is regulated.

For the purpose of the present invention however, a promoter is defined as the sequence extending over several hundreds of base pairs upstream of the coding region of the gene with the most important part situated in the 200 base pairs preceding the transcription initiation site. Examples of sequences that contain promoters from essential genes can be retrieved from GenBank. The complete sequence for EHV-1 and EHV-4 have been deposited at GenBank under Accession No. M86664

and AF030027, respectively. For genes 57 and 12 from EHV-1, the promoter will be located between nucleotides 101.600-102.347 and 12.900-13.505, respectively.

Alternatively, promoter regions can be determined by nucleotide sequence analysis of DNA fragments that contain an essential gene including the region upstream of the coding sequence.

A preferred embodiment of the invention is the promoter of the IE gene of EHV-1 of which one copy is located between nucleotide 118.590 and about 119.890 in the Inverted Repeat of the short segment (IRs).

It has been found according to the invention that the promoter region of essential genes can be more suitable for attenuating the virulence of a given virus. Until now this has not been considered because the manipulation of an essential gene was assumed to be deleterious for efficient replication in the host.

The term "essential gene" as used herein is intended to encompass genes that are obligatory for replication in the host. "Gene" is used solely for the coding sequence, whereas "promoter region" refers to the regulatory sequences necessary for expression of the gene.

Preferred mutations according to the invention are deletions between 1 and about 500 bases at any position within the promoter region that reduce the level of expression of the essential gene that is located downstream and attenuates virulence of the pathogen.

For EHV-1 preferred deletions are deletion of the SacI-SacI fragment or the HindIII-ClaI fragment or the NdeI-NdeI fragment or the SphI-SphI fragment of the promoter region of the Immediate Early gene.

As an alternative fragments with a size between 1 and about 500 bases can be inserted in the promoter region.

Substitutions can be made up to the complete promoter region provided that the substituted promoter is not a cell, tissue or host specific promoter as described

by Glazenburg et al. in US-5,580,564. Glazenburg aims at choosing the cell, the tissue and/or the host such that the harmful characteristics of the micro-organism are not expressed or are only expressed to an acceptable degree.

5 The overall virulence of the virus is not decreased, but instead the localization of the viral replication is limited to particular cells, tissues or hosts.

Substitutions of the complete promoter according to the invention are not intended to encompass such specific

10 promoters that lead to a modified cell, tissue or host tropism.

Deletions can be introduced by the following methods. Based on the map of the promoter region, suitable restriction sites can be deduced to remove

15 fragments of a defined size and at a defined position in the promoter. As an alternative one could start at a single site, and make progressive deletions in either one or two directions using the technique described by Henikoff (Gene 28, 351-359 (1984)). Also linker or PCR-

20 mediated mutagenesis (Current Protocols in Molecular Biology, Eds. Ausubel et al., Chapter 8, John Wiley & Sons. Inc. (1996) can be used.

Mutations introduced in cloned subfragments can be transferred to the virus genome as for example

25 described in Maniatis, T. et al. (1982) in "Molecular cloning", Cold Spring Harbor Laboratory; European Patent Application 74.808; Roizman, B. & Jenkins, F.J. (1987), Science 229, 1208; Higuchi, R. et al. (1988), Nucleic Acids Res. 16, 7351.

30 Briefly, this can be accomplished by constructing a recombinant DNA molecule for recombination with Equine herpesvirus DNA. Such a recombinant DNA molecule comprises vector DNA which may be derived from any suitable plasmid, cosmid, virus or phage, and

35 contains Equine herpesvirus DNA of the region identified above.

Examples of suitable cloning vectors are plasmid vectors such as pBR322, the various pUC and

Bluescript plasmids, cosmid vectors, e.g. THV, pJB8, MUA-3 and CosI, bacteriophages, e.g. lambda-gt-WES-lambda B, charon 28 and the M13mp phages or viral vectors such as SV40, Bovine papillomavirus, Polyoma and Adeno viruses.

- 5 Other vectors to be used can be retrieved from the Intelligenetics vector database accessible through website <http://www.seqnet.dl.ac.uk>.

- A deletion to be introduced in the described region can be incorporated first in a recombinant DNA molecule carrying the promoter region of the essential gene of EHV by means of a restriction enzyme digest with one or more enzymes of which the cleavage sites are correctly positioned in the promoter region of the gene. Recircularization of the remaining recombinant DNA molecule would result in a derivative lacking at least part of the promoter region. Alternatively, progressive deletions can be introduced either in one or two directions starting from within the restriction enzyme cleavage site present within the sequence of the gene.
- 15
- 20 Enzymes such as Bal31 or exonuclease III can be used for this purpose.

- Recircularized molecules are transformed into E. coli cells and individual colonies can be analyzed by restriction mapping in order to determine the size of the deletion introduced into the promoter region. An accurate positioning of the deletion can be obtained by sequence analysis.
- 25

- In case the insertion of a heterologous nucleic acid sequence is desired the recombinant DNA molecule comprising the EHV essential gene may be digested with appropriate restriction enzymes to produce linear molecules whereafter the heterologous nucleic acid sequence can be ligated to the linear molecules followed by recircularization of the recombinant DNA molecule.
- 30

- 35 Optionally, a deletion is introduced into the promoter region of the EHV gene concomitantly with the insertion of the heterologous nucleic acid sequence.

In case the method of in vivo homologous recombination is applied for the preparation of an EHV mutant according to the invention the EHV sequences which flank the deleted gene sequences or the inserted
5 heterologous nucleic acid sequences should be of appropriate length, e.g. 50-3000 bp, as to allow in vivo homologous recombination with the viral EHV genome to occur.

Subsequently, cells, for example equine cells
10 such as equine dermal cells (NBL-6) or cells from other species such as RK13, Vero and BHK cells can be transfected with EHV DNA in the presence of the recombinant DNA molecule containing the mutation flanked by appropriate EHV sequences whereby recombination occurs
15 between the EHV sequences in the recombinant DNA molecule and the corresponding sequences in the EHV genome.

Recombinant viral progeny is thereafter produced in cell culture and can be selected for example genotypically or phenotypically, e.g. by hybridization.
20 The selected EHV mutant can be cultured on a large scale in cell culture whereafter EHV mutant containing material can be collected therefrom.

The EHV-1 genome contains two copies of the Immediate Early gene, in opposite orientation. In this
25 case a convenient method for mutating the promoter is the construction of a set of cosmids harboring fragments of the EHV genome, including only one of the two copies of the IE gene and its promoter region. This single copy of the promoter region can then be mutated in a routine
30 manner. The set of cosmids is transfected to a confluent monolayer of host cells. Viruses will be formed by recombination and can be recovered from plaques formed in the monolayer. It is necessary to use one copy of the IE promoter region and corresponding gene for the
35 preparation of a mutant according to the invention to prevent replacement of the mutation by the non-mutated promoter region. The mutant virus will restore a second identical copy of the mutated promoter region of the IE

gene while assembling a functional full-size genome containing both inverted repeats flanking the Us.

It is standard procedure to test the efficacy and safety of a vaccine in vivo. Initial testing of
5 mutant EHV viruses can be performed in the well-known mouse model as described by van Woensel et al., J. Virological Methods 54, 39-49, (1995) and Osterrieder et al., Virology 226, 243-251, (1996). Final testing of the safety and efficacy of the virus strain takes place in
10 vivo in horses.

The mutation(s) in the promoter of one or more essential genes can be combined with one or more mutations in one or more other genes and/or their promoters. Examples of these are mutations creating a
15 marker vaccine which allow differentiation of vaccinated animals from infected animals. This way a further optimization of the vaccine's properties can be obtained.

The invention further relates to a nucleic acid sequence, comprising the promoter region of an EHV
20 essential gene and optionally one or more flanking sequences, which promoter region comprises one or more deletions, substitutions or insertions.

Like in the mutant, the nucleic acid sequence preferably comprises the endogenous promoter region of
25 the said essential gene into which deletions, substitutions or insertions are introduced, and preferably deletions.

Specific deletions are deletions of the SacI-SacI fragment or the HindIII-ClaI fragment or the NdeI-NdeI fragment or the SphI-SphI fragment of the promoter
30 region of the Immediate Early gene.

The nucleic acid sequence in a specific embodiment comprises the promoter region of EHV-1 or EHV-4.

35 The gene is for example an Immediate Early gene, or any other essential gene, such as those described hereinabove.

This nucleic acid sequence can be incorporated in a recombinant DNA molecule, which is also part of the invention. The selection of suitable vectors to prepare the recombinant DNA molecule are well within the reach of
5 the person skilled in the art.

In addition the invention relates to a host cell harboring such a recombinant DNA molecule, and to a vaccine comprising an EHV mutant of the invention and a pharmaceutically acceptable carrier or diluent.

10 A live attenuated mutant according to the present invention can be used to vaccinate animals, particularly domestic and non-domestic, and more specifically horses. Vaccination with such a live vaccine is preferably followed by replication of the mutant
15 within the inoculated host, which host will then elicit an immune response against EHV and the animal inoculated with the EHV mutant according to the invention will be immune to infection by EHV. Thus, a mutant according to claim 1 can serve as a live vaccine.

20 For the preparation of a live vaccine the recombinant EHV mutant according to the present invention can be grown on a cell culture, for example of equine, rabbit, hamster or calf origin. The viruses thus grown can be harvested by collecting the tissue cell culture
25 fluids and/or cells. The live vaccine may be prepared in the form of a suspension or may be lyophilized.

The vaccine according to the invention can be prepared using standard techniques available in the art. In general the vaccine is prepared by mixing the virus
30 with a pharmaceutically acceptable carrier or diluent.

For administration to animals, the EHV mutant according to the present invention can be given inter alia intranasally, intradermally, subcutaneously or intramuscularly.

35 Pharmaceutically acceptable carriers or diluents that can be used to formulate a vaccine according to the invention are sterile and physiologically compatible such as for example sterile

water, saline, aqueous buffers such as alkali metal phosphates (e.g. PBS), alcohols, polyols and the like. In addition the vaccine according to the invention may comprise other additives such as adjuvants, stabilizers, 5 anti-oxidants, preservatives and the like.

Suitable adjuvants include but are not limited to aluminum salts or gels, carbomers, non-ionic blockcopolymers, tocopherols, monophospheryl lipid A, muramyl dipeptide, oil emulsions (w/o or o/w), cytokines. 10 The amount of adjuvant added depends on the nature of the adjuvant itself.

Suitable stabilizers for use in a vaccine according to the invention are for example carbohydrates including sorbitol, mannitol, starch, sucrose, dextrin 15 and glucose, proteins such as albumin or casein, and buffers like alkaline phosphates.

Suitable preservatives include, amongst others, thimerosal, merthiolate and gentamicin.

Live vaccines according to the invention 20 comprise an effective amount of the afore-mentioned EHV mutant virus and a pharmaceutically acceptable carrier. The term "effective" as used herein is defined as the amount sufficient to induce an immune response in the target animal. The amount of virus will depend on the 25 route of administration, the time of administration as well as age, general health and diet of the subject to be vaccinated.

The dosages in which the live vaccines according to the invention can prevent infectious disease 30 can be readily determined by routine trials with appropriate controls and are well within the routine skills of the practitioner.

The useful dosage to be administered will vary depending on the age, weight, mode of administration and 35 type of pathogen against which vaccination is sought. A suitable dosage can be for example about $10^{3.0}$ - $10^{7.0}$ pfu/animal.

According to a further aspect thereof the invention provides a process for the preparation of EHV mutants, comprising transfecting a cell culture with a recombinant DNA molecule and EHV genomic DNA.

5 The principle of mutating an essential gene of the pathogen can also be used in a broader sense for other herpesviruses, such as bovine herpesvirus, pseudorabies virus, feline herpesvirus. Marek's disease virus, infectious laryngotracheitis virus etc.. The
10 invention thus provides for a method of genetically attenuating herpesviruses, comprising mutation of the promoter region of an essential gene, which mutation consists of one or more deletions, substitutions or insertions in the promoter region of an essential gene.
15 Preferably, the deletions, substitutions or insertions are introduced into the endogenous promoter region of the said essential gene.

The present invention will be further illustrated in the non-limiting examples that follow. All
20 molecular biological techniques that are used in the examples are standard procedures as for example described in Sambrook et al., "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Laboratory Press (1989). These techniques will not be described in detail.

25 In the Examples reference is made to the following figures:

Figure 1 shows a map of the EHV-1 genome with the position of five cosmid DNA inserts that restore a viable virus after contransfection and recombination
30 between their overlapping sequences. Deletions in the IE gene promoter were introduced in a 8 kbp SpeI fragment of the IRs that was re-assembled with a 21 kbp SpeI and used as such to replace cosmid 2D9 in the cotransfection experiment.

35 Figure 2 shows a schematic layout of the promoter region of the IE gene in the IRs from EHV-1. Various sequence elements that may regulate transcription initiation are indicated. Below are shown the deletions

that were introduced and the mutants that were generated by incorporating these deletions into the viral genome after co-transfection with the set of overlapping cosmid DNA inserts. Strain EHV24 was restored in a similar
5 manner but contained no deletions in the promoter region.

EXAMPLES

EXAMPLE 1

10 Construction of the cosmid set for generating EHV-1 virus

The SuperCos 1 cosmid vector kit was purchased from Stratagene (Catalog# 251301) and enzymes were purchased at New England Biolabs. The vector was further modified by adding extra restriction enzyme sites to it.

15 For this a DNA linker was purchased from Pharmacia containing the following restriction sites: BamHI, I-SceI, PacI, AscI, EcoRV, PacI, AscI, I-SceI and BamHI. The SuperCos 1 vector and the linkers were both cut with BamHI. The BamHI digested vector was dephosphorylated
20 with alkaline phosphatase after which the BamHI digested linker was ligated into the SuperCos 1 vector by T4 DNA ligase, all according to the manufacturer's instructions. The resulting vector was designated THM and used for cloning the EHV-1 inserts.

25 Viral DNA was obtained from the EHV-1 M8 strain, a pathogenic EHV-1 strain isolated from a horse with severe signs of an EHV-1 infection, but could be any pathogenic EHV-1 strain. This particular virus was grown in cultures of Vero cells and was recovered by
30 centrifugation. The viral DNA was prepared by lysis with EDTA and SDS, phenol extraction and alcohol precipitation.

The first set of cosmids was constructed by digestion of the EHV-1 DNA with PacI. After phenol
35 extraction of the M8 PacI digests, the ends were filled in with T4 DNA polymerase and then dephosphorylated with alkaline phosphatase. The cosmid vector THM was digested

with EcoRV and the inserts were ligated into the vector with T4 DNA ligase.

The ligation mix was added to a packaging mix (Gigapack, Stratagene) according to the manufacturers instructions. The packaged DNA was added to a fresh overnight culture of *E.coli* DH1 and placed for 1 hour at 37°C. The bacteria suspension was then spread onto agar plates containing ampicillin. Plasmid DNA from all colonies was submitted to restriction enzyme analysis on the insert.

For the construction of other cosmids the same procedure was followed only now the viral DNA was digested with AscI, AseI, RsrI, or NotI, all ends were then filled in with T4 DNA polymerase and the inserts ligated into the EcoRV site of the vector.

To obtain a third generation of cosmids the viral DNA was sheared twice through a 19G needle, the ends were then filled in with T4 DNA polymerase and after phenol extraction and precipitation the inserts were cloned again into the EcoRV site of the cosmid vector THM. The vector with the inserts was then packed, put on bacteria and the colonies analyzed.

From all colonies obtained the restriction maps were determined by multiple digestions. Then the location of the different clones was determined by comparing the restriction map of the clones with the restriction map of EHV-1 strain Ab4 (Telford et al, 1992).

Cosmids 2D3, 1A12, 1F4, 2C12 and 2D9 were selected such that the complete genome could be reconstituted by recombination between overlapping sequences (Fig. 1).

Viable virus was generated after transfecting EHV-1 inserts excised from the cosmids by digestion with I-SceI (Boehringer) into cultures of equine dermal cells (ATCC number CCL-57) using the calcium phosphate method. Viral progeny was passaged and plaque purified on monolayers of rabbit kidney (RK13, ATCC number CCL-37) or other kidney cells that support viral replication.

EXAMPLE 2Introducing deletions in the promoter of the IE gene

The genome of EHV-1 contains two copies of the Immediate Early (IE) gene located in the repeated sequence elements IRs and TRs flanking the unique short region. Stable mutations affecting expression of the IE gene need to be introduced at both positions simultaneously before the new phenotype will be expressed.

Therefore, one of the cosmids named "2D9" was modified such that a fragment of about 7 kbp at the right end of the TRs and containing a single copy of the IE gene, was deleted. This was obtained by subcloning the two SpeI fragments of 8 and 21 kbp from 2D9 in pGEM-9Zf(-) (Promega) using the SpeI or SpeI/XbaI sites, respectively (Fig. 1). Insertion of the 21 kbp fragment resulted in pEHV17 and restored a single SpeI site in which the 8 kbp SpeI fragment subsequently can be inserted to regenerate a 29 kbp EHV-1 fragment used for replacing 2D9 in the cosmid transfection described in Example 1. The 8 kbp SpeI contains all of the coding sequence of the IE gene and about 2 kbp of upstream sequences including the promoter of the IE gene. Mutations in the promoter region were introduced in a plasmid designated pEHV06 that contained a 2 kbp subfragment from the 8 kbp SpeI. This insert included a 1 kbp EcoRV fragment which essentially represented the promoter and other regulatory elements located in the sequence upstream of the coding region of the IE gene (Fig. 2).

By selecting the proper restriction enzymes that had two site in the EcoRV fragment of pEHV06 and relatively near to each other, small deletions with an exact size could be introduced at various positions in the promoter sequence of the IE gene. The enzymes SacI, NdeI, SphI and a combination of ClaI and HindIII were suitable for this purpose. The deletions introduced in pEHV06 by digesting with each of these enzymes and

recircularizing the plasmid DNA with T4 DNA ligase are represented in Fig. 2.

The mutated EcoRV inserts were exchanged with the original EcoRV insert in the 8 kbp SpeI fragment and these were subsequently assembled with the 21 kbp SpeI in pEHV17 resulting in plasmids that were designated pEHV22, pEHV23, pEHV25 and pEHV26 for the deletions generated with the enzymes SacI, HindIII/ClaI, NdeI and SphI, respectively (Fig. 2). pEHV24 contained the re-assembled 8 and 21 kbp SpeI fragments but without any deletion in the promoter region. These plasmids were used to replace 2D9 in the cotransfection of equine dermal cells with the cosmid inserts from 2D3, 1A12, 1F4 and 2C12, generating viable virus with defined small deletions in the promoter region of both copies of the IE gene. Viral progeny was plaque purified and amplified on RK13 cells. Presence and size of the deletions were confirmed by DNA blot analysis and PCR.

Mutant strains were designated EHV22, EHV23, EHV25 or EHV26 with the number corresponding to the plasmid used in the cotransfection experiment. Strain EHV24 represents the control virus and contains the non-mutated promoter region.

Cosmid derived mutant EHV strains 22, 25 and 26 and the control strain EHV24 grow to titers between 2 and 4×10^6 pfu/ml on RK13 cells, which is slightly less than the parent M8 strain that grows to titer of 5×10^6 pfu/ml. Strain EHV23 replicated less efficiently and resulted in titers of about 2×10^5 pfu/ml.

EXAMPLE 3

Analysis of virulence in mice

Infection with EHV-1 can cause pyrexia and clinical signs of respiratory disease but most important is the induction of abortion. However, seen the complexity of vaccination trials in pregnant horses, models have been developed in mice measuring the pathogenicity of EHV-1 strains based on weight reduction

(van Woensel et al., J. Virological Methods 54, 39-49, (1995); Osterrieder et al., Virology 226, 243-251, (1996).

Mice that are 4-5 weeks of age are inoculated intranasally with a dose of $10^{6.5}$ pfu per animal. Body weights are determined on the 9 days following infection and the weight gain as percentage of the weight at day 0 are plotted in a graph. The degree of pathogenicity of various EHV-1 strains is deduced from the position of resulting curves relative to each other. Significant losses in body weight, particularly at day 3 and 4 after infection, correlate with high virulence of the strain of interest. Series of EHV-1 isolates carrying deletions in the IE promoter region (see Example 2) can be analyzed for virulence by comparison with the parent M8 strain or cosmid generated EHV-1 virus containing the intact promoter sequence.

EXAMPLE 4

20 Analysis of virulence in horses

Although pathogenicity of individual EHV-1 strains using the mouse model can be correlated with behavior in the natural host, more conclusive evidence can be obtained from vaccination trials in horses. In addition, the level of protection against challenge infection and ultimately in preventing abortion in pregnant mares can only be established in the target animal.

Horses at the age of about one year and previously tested for the absence of EHV-1 serum antibodies, are vaccinated intranasally with a dose between 10^5 and 10^6 pfu.

Optionally, animals can be revaccinated 4 to 6 weeks after the primary vaccination. Animals are monitored daily for signs of clinical disease and replicating virus in samples from mucus, urine, faeces, ocular secretions and vaginal swabbings are assayed during the 14 days following vaccination. Viremia are

detected by plating blood leukocytes on monolayers of RK cells.

Major safety features for a live EHV-1 vaccine strain are the stability upon serial passaging in vitro 5 and in vivo, and the absence of any signs of clinical disease after administration to the host. In addition, the virus should only replicate for a limited time in the upper respiratory tract and should not become viremic or detectable in the nervous system. Ideally it should not 10 be shed from vaccinates to naive in-contact animals.

The efficacy of the vaccine is measured by challenging the animals with a virulent EHV-1 isolate. Attenuated strains that are able to establish a solid protection against EHV-1 will prevent the challenge virus 15 to become viremic and therefore minimize the risk of placental infection and abortion of the foetus. Strains that fulfil these requirements are selected from candidates that were constructed as described in Example 2.

20 Other strains of EHV-1 that are non-pathogenic due to similar deletions in the promoter of the IE gene may equally well be considered.

CLAIMS

31. 07. 1998

1. Equine herpesvirus (EHV) mutant, comprising one or more deletions, substitutions or insertions in the promoter region of an essential viral gene.
2. EHV mutant as claimed in claim 1, wherein
5 the deletions, substitutions or insertions are introduced into the endogenous promoter region of the said viral gene.
3. EHV mutant as claimed in claim 2, wherein deletions are introduced into the promoter.
- 10 4. EHV mutant as claimed in claims 1-3, wherein the gene is the Immediate Early gene.
5. EHV mutant as claimed in claims 1-4, wherein the mutant virus is the EHV-1 virus or the EHV-4 virus.
6. EHV mutant as claimed in claims 1-5, further
15 comprising one or more mutations in one or more other genes and/or their promoters.
7. EHV-1 mutant as claimed in claims 1-6, comprising a deletion of the SacI-SacI fragment or the HindIII-ClaI fragment or the NdeI-NdeI fragment or the
20 SphI-SphI fragment of the promoter region of the Immediate Early gene.
8. Nucleic acid sequence, comprising the promoter region of an essential gene from EHV and optionally one or more flanking sequences, which promoter
25 region comprises one or more deletions, substitutions or insertions.
9. Nucleic acid sequence as claimed in claim 8, comprising the endogenous promoter region of the said essential gene into which deletions, substitutions or
30 insertions are introduced.
10. Nucleic acid sequence as claimed in claim 9, comprising the endogenous promoter region of the said essential gene into which deletions are introduced.
11. Nucleic acid sequence as claimed in claims
35 8-10, wherein the gene is an Immediate Early gene.

12. Nucleic acid sequence as claimed in claim
10 or 11, comprising a deletion of the SacI-SacI fragment
or the HindIII-ClaI fragment or the NdeI-NdeI fragment or
the SphI-SphI fragment of the promoter region of the
5 Immediate Early gene.

13. Nucleic acid sequence as claimed in claims
8-12, wherein the EHV is EHV-1 or EHV-4.

14. Recombinant DNA molecule comprising a
nucleic acid sequence as claimed in claims 8-13.

10 15. Host cell harboring a recombinant DNA
molecule as claimed in claim 14.

16. Vaccine comprising an EHV mutant as claimed
in claims 1-7 and a pharmaceutically acceptable carrier
or diluent.

15 17. A process for the preparation of an EHV
mutant as claimed in claims 1-7, comprising transfecting
a cell culture with a recombinant DNA molecule as claimed
in claim 14 and EHV genomic DNA.

18. Method of genetically attenuating
20 herpesviruses, comprising mutation of the promoter region
of an essential gene, which mutation consists of one or
more deletions, substitutions or insertions in the
promoter region of an essential gene.

19. Method as claimed in claim 18,
25 characterized in that the deletions, substitutions or
insertions are introduced into the endogenous promoter
region of the said essential gene.

20. Method as claimed in claim 18 or 19,
wherein the micro-organism is a herpesvirus selected from
30 the group consisting of equine herpesvirus, bovine
herpesvirus, feline herpesvirus, Marek's disease virus,
infectious larynchotracheitis virus, pseudorabies virus.

21. Method as claimed in claim 20, wherein the
herpesvirus is a Equine herpesvirus (EHV).

35 22. Method as claimed in claim 21, wherein the
EHV is EHV-1 or EHV-4.

23. Method as claimed in claims 18-22, wherein
the gene is an Immediate Early gene.

1/2

EPO - DG 1

31. 07. 1998

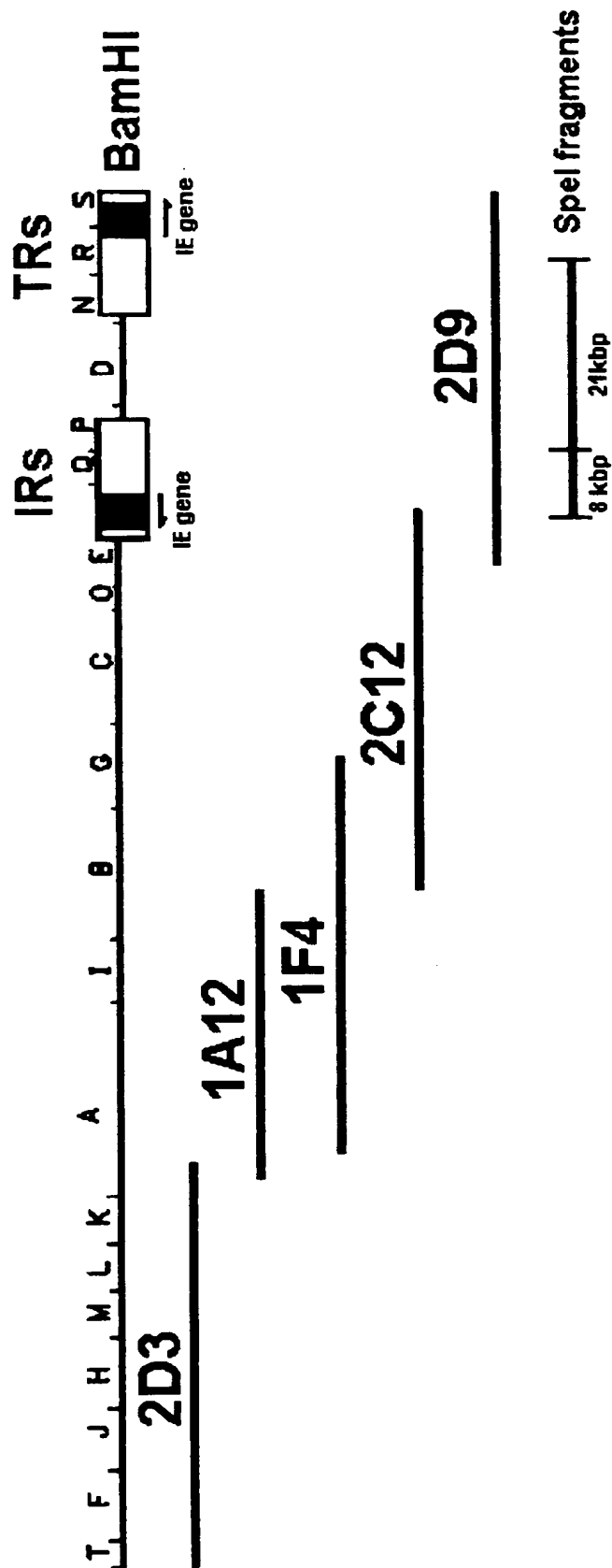


FIGURE 1

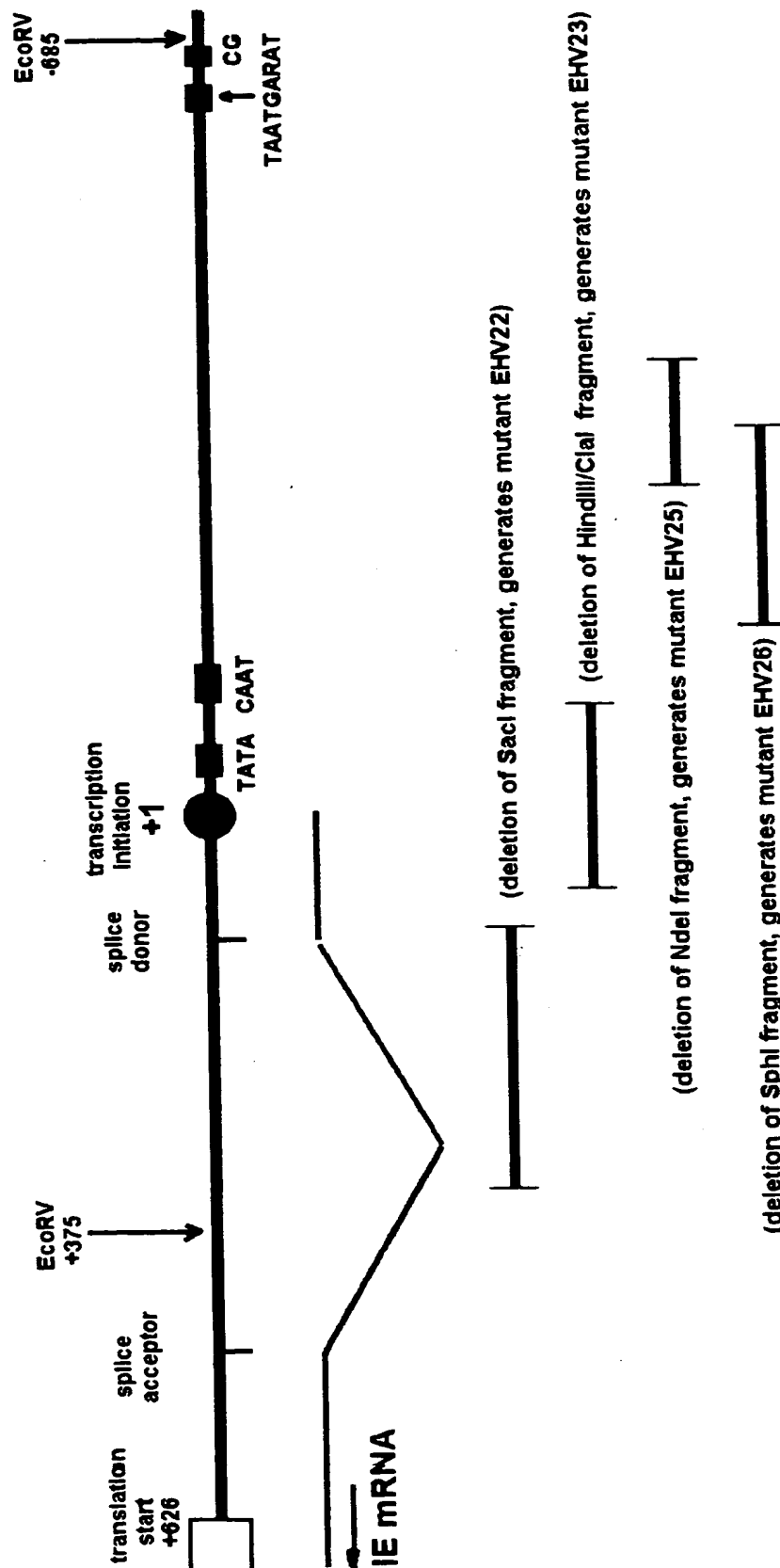


FIGURE 2